Proc. Natl. Acad. Sci. USA
Vol. 81, pp. 616–619, January 1984
Neurobiology

Rod light response augmented by active phosphodiesterase

(sensory transduction/vertebrate scotopic receptors/receptor potential/light-dependent channels)

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Communicated by Floyd Ratliff, September 19, 1983

ABSTRACT **Light activates rod outer segment (ROS) phosphodiesterase (PDEase), as shown by previous biochemical and physiological studies. We have further investigated the role of PDEase in this system by injecting trypsin-activated PDEase, purified from bovine ROS, into single ROS of the isolated retina of the toad *Bufo marinus*. Injection of about 300 molecules of activated PDEase in darkness is without immediate detectable effect, as measured by intracellular membrane-voltage recording. The effect of the activated PDEase injections only becomes evident after illumination. The light response is augmented; kinetics of repolarization are slowed. We conclude that this augmentation of the light-dependent hyperpolarization results from the hydrolysis of endogenous cyclic GMP caused by injected PDEase. These results provide evidence that PDEase affects light-dependent channels of the vertebrate scotopic photoreceptors but do not specify whether the effects are exercised for the initial hyperpolarizing phase of the receptor potential and for the recovery phase or only for the recovery phase.**

Light-mediated hydrolysis of cyclic GMP has been proposed as an intermediate step in phototransduction in the vertebrate retinal photoreceptors for dim light, the rods. Rod outer segments (ROS) contain a light-activated phosphodiesterase (PDEase) specific for cyclic GMP (1). A single photon absorbed by a single molecule of rhodopsin can lead to the activation of as many as 500 molecules of PDEase to hydrolyze $>10^5$ molecules of cyclic GMP per second (2). This high degree of amplification is mediated by an intermediate protein, transducin, which carries the activation signal from photolyzed rhodopsin to the PDEase (3).

Purified PDEase from ROS consists of a complex of α, β, and γ subunits, 88, 84, and 11 kilodaltons (kDa) in size (1). Trypsin activates the PDEase by selectively degrading the γ inhibitory subunit, leaving the α and β subunits intact (4).

What is the role of cyclic GMP in transduction? Iontophoretic injections of cyclic GMP depolarize the ROS and increase the latency of the light-induced receptor potential as if light-mediated cyclic GMP hydrolysis is necessary for phototransduction (5, 6). Light-induced cyclic GMP-PDEase activity is vigorous enough and its activation is rapid enough to mediate the earliest phases of rod transduction (2, 7, 8). Protons released during hydrolysis of cyclic GMP also may regulate the receptor potential (9). However, Ca^{2+} can regulate the ROS membrane potential, too. High Ca^{2+} concentrations not only mimic the receptor potential (10) but also lower intracellular cyclic GMP concentration (11). On the other hand, cyclic GMP can regulate the Ca^{2+} distribution within the cell by stimulating Ca^{2+} uptake by disks (12).

We have injected purified, trypsin-activated PDEase into single ROS of the isolated toad retina to observe physiological effects of increased hydrolysis of endogenous cyclic GMP. We find that such injections augment the light-induced receptor potential.

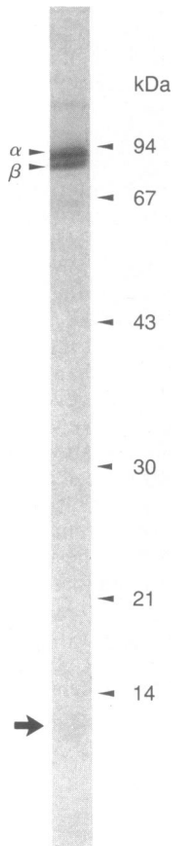

FIG. 1. $NaDodSO_4$/polyacrylamide gel electrophoresis of trypsin-activated bovine retinal PDEase used for injection experiments. Nine micrograms of activated PDEase was electrophoresed on $NaDodSO_4$/polyacrylamide gel made from 18% acrylamide/0.096% bisacrylamide. The positions of standard proteins are marked. The arrow indicates the position to which the 11-kDa γ inhibitor subunit has migrated in an adjacent lane. PDEase was extracted from bovine ROS as described (4). It was purified by HPLC gel filtration on TSK 3000SW and concentrated. The γ subunit was removed by digestion with trypsin at 0°C (4), activating the PDEase. The PDEase was then rechromatographed on the HPLC gel filtration column.

METHODS AND MATERIALS

Cyclic GMP PDEase was extracted from bovine ROS membranes, partially purified by gel filtration, activated by trypsin, and purified to near homogeneity (Fig. 1) by gel filtration

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Abbreviations: PDEase, phosphodiesterase; ROS, rod outer segment(s); kDa, kilodaltons.

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with a specific activity that ranged from 500 to 1,500 μmol/min per mg assayed at 20°C (4). Bovine ROS PDEase injected into toad ROS should be functional because PDEase isolated from retinas of a variety of species is indistinguishable with respect to molecular weight and immunologically reactive groups (13).

Purified activated PDEase was injected into ROS of the isolated retinas of the toad *Bufo marinus* using pressure applied to the recording glass micropipette. The pipettes contained ≈0.5 μl of purified activated PDEase in 5 mM or 50 mM Tris·HCl buffer (pH 8.0) or purified activated PDEase in 20 mM 3-(*N*-morpholino)propanesulfonic acid/30 mM NaCl/60 mM KCl/1 mM dithiothreitol/100 μM phenylmethylsulfonyl fluoride, pH 7.5. The concentration of activated PDEase in the pipettes ranged from 0.05 to 1.8 mg/ml. The upward-going spikes on the signal traces below the records indicate light flashes illuminating the entire retinal patch in the chamber with about 200 effective photons per flash per rod. Down on the signal traces indicates the time during which pressure is applied to the pipette.

RESULTS

Injected PDEase Augments Light-Dependent Hyperpolarization. Fig. 2*A* shows responses to three light flashes, each containing about 200 photons absorbed per rod. The second flash is preceded by the 60-sec application of 7 bars (1 bar = 100 kPa) to the recording pipette, which contains 2.5 μM activated PDEase in 50 mM Tris·HCl (pH 8.0). The initial hyperpolarizing phase caused by the first and last light flashes is followed by a recovery phase lasting about 5 sec. The response to the light flash delivered just after the PDEase injection is augmented. The recovery phase is extended to about 3 min. The responses labeled 1 and 2 are normalized, superimposed, and expanded in Fig. 2*C*. Response 2 has a normal recovery time, even though the ROS is still hyperpolarized after the PDEase injection. Response 1 is expanded in Fig. 2*B* to show that the depolarization caused by the PDEase injection in darkness has recovered before the light flash is delivered.

Brief pressure injection pulses (Fig. 3) cause more pronounced effects than prolonged injections (Fig. 2), possibly because the pipette tip is more subject to clogging from prolonged pressure. Each brief pulse causes spike-like artifacts, as shown on Fig. 3. After the train of 300-msec, 7-bar injections of PDEase (Fig. 3*A*), the light flash causes a response that shows little recovery. The membrane potential continues to hyperpolarize to an absolute level of −57 mV after the light response following the PDEase injections (Fig. 3*A*) and shows no recovery in the further 10 min the cell is held, possibly reflecting the large amount of active PDEase injected in this case. There are three types of controls: 1, the same intensity light flash is delivered before and after the injection as in Fig. 3*A*; 2, the same pipette is heated to 60°C for 15 min to inactivate the PDEase and used for injections into other ROS. No such injections augmented the light response, but it is impossible to know whether the inactivated enzyme is actually injected into the cell; 3, the buffer vehicle alone is injected as in Fig. 3*B*, where the pipette is loaded with 50 mM Tris·HCl. The train of 500-msec, 8-bar pulses causes a reversible depolarization without affecting the light response that follows the injections.

Fig. 4*A* illustrates the effect of a single 200-msec, 4-bar PDEase injection. Recovery of the light response after the PDEase injection labeled 2 takes about 2 min compared to 5 sec for the controls before and after the injection. Responses 1 and 2 are expanded and superimposed on the trace in Fig. 4*B* to illustrate again (as in Figs. 2*B* and 3*C*) that there is recovery to the base line after the PDEase injection. Thus, the augmentation of the light response does not represent an artifactual hyperpolarizing undershoot or rebound from the

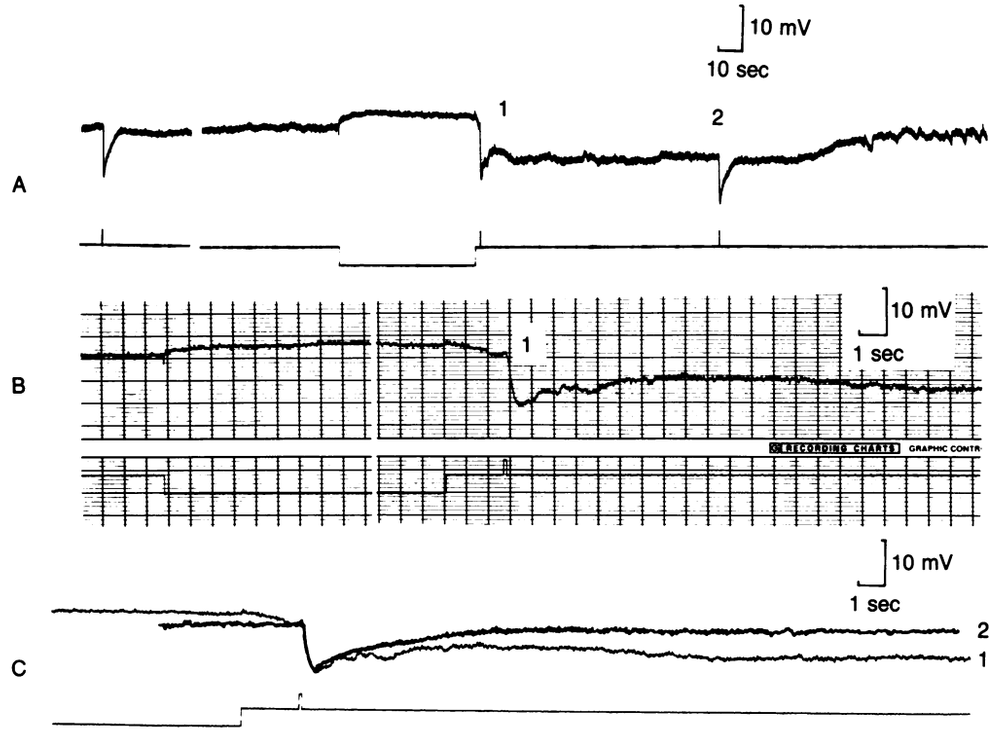

FIG. 2. (*A*) Sixty-second, 7-bar injection of 2.5 μM purified, active PDEase into single ROS. Injection is preceded by a control light flash and followed by light flashes labeled 1 and 2. Recovery of response 1 is inhibited without affecting response 2; (*B*) response 1 is expanded to show that depolarization during injection recovers before light response commences; (*C*) responses 1 and 2 are expanded, normalized, and superimposed to illustrate inhibition of recovery of response 1 after PDEase injection. Light flash intensities were about 200 effective photons per ROS in *A–C*. Up on signal trace indicates light; down indicates pressure injection.

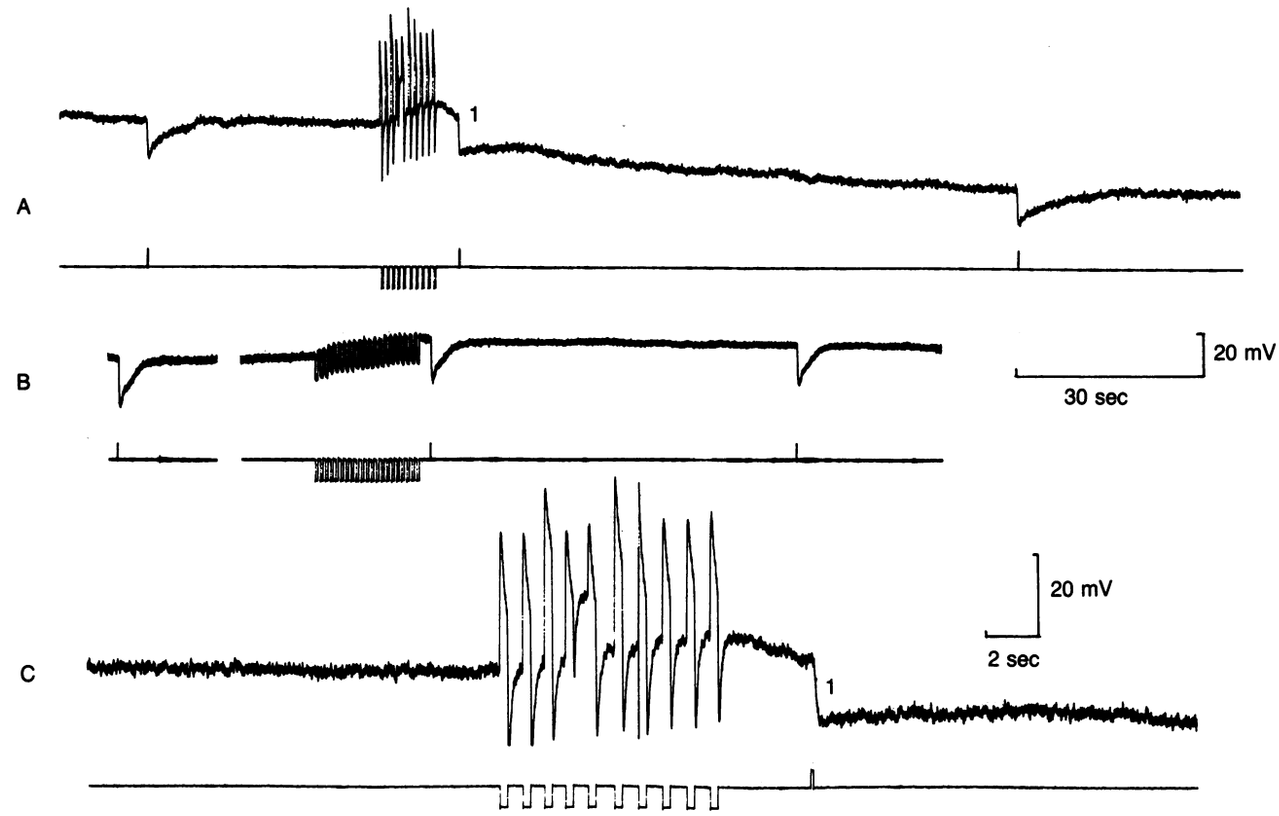

FIG. 3. Comparison of effect of train of 300-msec, 7-bar injections of purified 2.5 μM PDEase in 50 mM Tris·HCl buffer (pH 8) on light response (*A*) with effect of train of 500-msec, 8-bar injections of buffer vehicle alone (*B*). Thirty seconds is excised from the trace in *B* between the first flash and injections of buffer. (*C*) PDEase injections expanded to show recovery from depolarization during injections before light response. Flash intensities were the same as for Fig. 2. Large spike transients during injections are artifacts.

pressure injection (neither do 300-msec pressure injections of cyclic GMP, which cause transient depolarizations antagonized by light, cause any hyperpolarization undershoot). The light flash that elicits the prolonged light-dependent hyperpolarization in Fig. 4 is delivered 7 sec after the 200-msec PDEase injection and after recovery from the instability caused by the injection (Fig. 4*B*). Seven seconds is sufficient time for the injected PDEase to be incorporated into the system and exert a light-dependent effect without causing a detectable hyperpolarization in darkness. The base line can be followed between pulses in the train in Fig. 3*A*. No sign of a hyperpolarization is seen in the 12 sec before the flash in Fig. 3*A* that causes an 18-mV hyperpolarization lasting for minutes. Similarly, only a small depolarization is observed during the 60-sec PDEase injection shown in Fig. 2. In contrast to the lack of immediate detectable hyperpolarization attributable to injection of active PDEase in darkness, we observe augmentation of light-dependent hyperpolarizations in 16 cells after the injection of purified activated PDEase, regardless of the buffer vehicle.

DISCUSSION

PDEase Appears to Affect Light-Dependent Channels. We find that injection of trypsin-activated PDEase prolongs the recovery phase of the voltage response to light. That injected PDEase affects the light response is evidence that PDEase is acting through the same molecular pathway as light. Yet, we find these results to be puzzling and unexpected—puzzling because the observations do not fit our preconception that

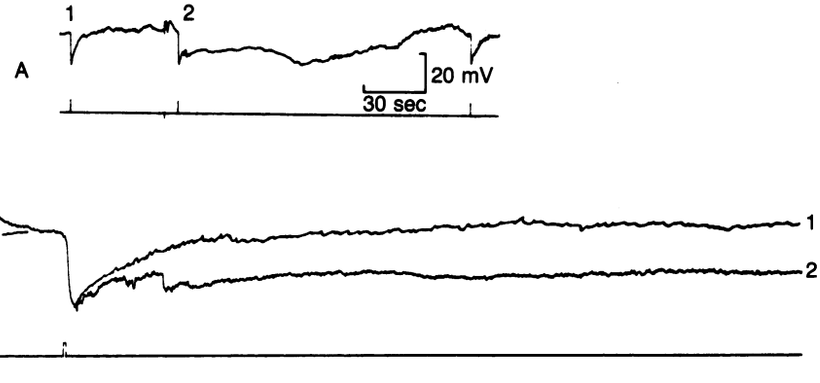

FIG. 4. (*A*) Single 200-msec, 4-bar injection of purified activated PDEase into ROS produces an effect similar to that described for Figs. 2 and 3. (*B*) Responses 1 and 2 expanded to show effect of PDEase injection and illustrate that instability caused by the injection has subsided before light response 1 commences. Light flash intensities and signal traces are described in the legend to Fig. 2.

active PDEase would be expected to hydrolyze cyclic GMP and directly lead to rapid hyperpolarization and unexpected because a previous study (14) reports a reversible hyperpolarization during the injection in darkness of "partially purified, partially activated PDEase" and no prolongation of the receptor potential. We cannot explain the difference between this previous report (14) and our results reported here.

Relation to Transduction Hypotheses. There are four major transduction hypotheses:

Hypothesis 1. Ca^{2+} is released by light to block Na^+ conductance. The mechanism of Ca^{2+} release by light is not specified in this model. Cyclic GMP functions only to mediate recovery after illumination by stimulating Ca^{2+} uptake. Light-activated PDEase slows the rate of Ca^{2+} uptake and recovery (12).

Hypothesis 2. Cyclic GMP acting through a protein kinase controls the state of phosphorylation of the Na^+ pore. Light-activated PDEase lowers cyclic GMP to allow dephosphorylation activity of phosphatase to block Na^+ conductance (15).

Hypothesis 3. Light-activated PDEase produces protons via cyclic GMP hydrolysis. Protons exchange for Ca^{2+} which blocks the Na^+ conductance (9).

Hypothesis 4. A combination of hypotheses 2 and 3.

Both the failure to detect an immediate hyperpolarization in the dark and the prolongation of the light response are consistent with all of the hypotheses, assuming that a small amount of PDEase is injected. We estimated the amount of PDEase injected by comparing the effects of pressure injection of cyclic GMP to iontophoretic injection of cyclic GMP. We find that 300-msec, 4-bar injections of 25 mM cyclic GMP produce results equivalent to 5-pC current injections. A 5-pC current pulse was previously estimated to increase the dark ROS cyclic GMP concentration (6) by a third. This is an estimate of the upper bound based on a transference number of 1. The transference number for cyclic GMP is more likely lower—e.g., 0.1. A 5-pC iontophoretic injection then increases the dark ROS cyclic GMP concentration by about 3%. Thus, a 200-msec, 4-bar injection of PDEase, using a pipette loaded with 2.5 μM PDEase as in Fig. 4, corresponds to an injected volume of 0.007% of the ROS volume or about 300 molecules of PDEase.

One-picocoulomb iontophoretic pulses of cyclic GMP, which may increase ROS cyclic GMP concentration by 0.6%, cause transient depolarizations lasting about a second (6). Cyclic GMP depolarizations in more dark-adapted preparations can be an order of magnitude slower, which implies that both the dark hydrolysis and synthesis (cyclase) rates may be about 9 μM/sec. An additional 300 active PDEases would increase the dark rate by 8% and initially decrease cyclic GMP concentration at a rate of 0.7 μM/sec, based on a PDEase turnover number of 4,000 mol of cyclic GMP per mol of PDEase (4). The dark cyclic GMP concentration would be reduced from 50 μM (16) to 44 μM in something over 10 sec. The action of light would rapidly bring cyclic GMP levels to the new steady state. The new steady state would be prolonged while the return to normal dark cyclic GMP concentration is postponed by the active PDEase, which cannot be deactivated. A light response after the cell has reached the altered steady state, as in (*a*) response 2 of Fig. 2 and (*b*) the last response in Fig. 3*A*, would be followed by a normal recovery to the same steady-state concentration of cyclic GMP. Still, the lack of any immediate sign of hyperpolarization after PDEase injections is not explained by the steady-state model, although it is possible that the depolarization artifact caused by injection tends to mask any slow hyperpolarization. However, the failure to hyperpolarize in darkness could be made consistent with hypotheses 2–4 by assuming PDEase inactivation by excess inhibitor (4, 17), only to be activated by illumination. Against this postulate we find that purified PDEase assayed with homogenized ROS in darkness in the ratio of purified PDEase to PDEase in homogenized ROS of 1:20 and 1:200 does not significantly alter enzymatic activity.

In the event that large amounts of active PDEase were injected and the ROS cyclic GMP were to have been rapidly and severely depleted without any physiological sign of hyperpolarization, the failure to hyperpolarize in the dark would only be consistent with hypothesis 1.

Conclusion. Purified activated PDEase, injected into ROS, augments the light-dependent hyperpolarization. Whatever the mechanism by which the injected PDEase acts, its action of augmenting the effects of light provides further evidence that light-activated PDEase is part of the endogenous transduction machinery.

We are indebted to Professor Lubert Stryer (Stanford University School of Medicine) and Professor E. N. Pugh, Jr. (University of Pennsylvania) for painstaking reviews, to Professor Melvin Simon (California Institute of Technology) for his support, and to Rosemary Hopkins for technical assistance. This work was supported in part by Public Health Service Grants AI-19296 (M. I. Simon, California Institute of Technology) and EY-03196 (W.H.M.) and by fellowships from Fight for Sight, Inc., New York City (Y.S.) and the Alexander B. Cox Memorial Fund, Yale Medical School (Y.S.).

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